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Genetic and Morphological Variation in *Callicarpa japonica* Thunb. (Lamiaceae) in Japan

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Callicarpa japonica (Lamiaceae) is a morphologically variable shrub widely distributed throughout Japan. In this study, we investigated the intraspecific genetic structure and geographical distribution pattern of the morphological variations. A border of genetic variation in the chloroplast DNA and nuclear rDNA ITS regions between eastern and western Japanese populations was recognized in the Kinki area. Leaf size and thickness varied continuously and the observed pattern of morphological variation was not coincident with genetic differentiation. Moreover, plants with large and/or thick leaves were found in both the eastern and western populations, indicating that they were not monophyletic.

Key words: *Callicarpa japonica*, chloroplast DNA, ITS, molecular phylogeny, morphological variation, phylogeography

Callicarpa japonica Thunb. (Lamiaceae) is a representative deciduous shrub in the temperate forests of Japan, Korea, Taiwan, and China. It is famous for its beautiful purple fruit and is often cultivated in gardens for ornamental use. It is also frequently found in lower mountain forests in Japan. The species is known to be morphologically variable, and several infraspecific taxa have been described (Hara 1948, Kitamura & Murata 1971, Murata 1989, Yamazaki 1993). Recently, two varieties, *japonica* and *luxurians* Rehder, have been recognized (Yamazaki 1993). Variety *luxurians* can be distinguished from var. *japonica* by its larger leaves. It occurs on the edges of coastal evergreen forests in Honshu (Izu Islands), southern Shikoku, central to southern Kyushu, and the Ryukyu islands, Japan, as well as on Lanyu Island in Taiwan (Yamazaki 1993). The type locality is on Cheju Island, Korea. Additionally, var. *luxurians* tends to have larger, thicker leaves and larger inflorescences than var. *japonica* (Kitamura & Murata 1971, Murata 1989). However, because of the large morphological range in leaf size, which varies continuously (Murata 1989), the two varieties cannot be clearly distinguished. Moreover, in addition to the populations with relatively large leaves in the southwestern region, individuals of *C. japonica* with large leaves, often identified as var. *luxurians*, occur in the Hokuriku and Tohoku regions in northeastern Japan (Iwate Shokubutsu-no-kai 1970, Horikawa 1972, Yuhki 1972, Ishikawa Shokubutsu-no-kai 1983, Ohta *et al.* 1983, Yuhki 1992), causing further taxonomic confusion.

Therefore, to elucidate the underlying genetics of the morphological variation and to alleviate taxonomic confusion, we examined the intraspecific genetic structure and the geographical distribution pattern of the morphological variations of *Callicarpa japonica* in Japan.

Materials and methods

Plant materials

We sampled 213 plants from 68 populations of *Callicarpa japonica* across Japan, from Hokkaido to Okinawa, and on Cheju Island, Korea (Table 1, Fig. 1), covering most of the range of distribution of vars. *japonica* and *luxurians* in Japan. We did not distinguish between varieties during sampling and analysis. We used 58 plants from 55 populations for molecular analysis. In addition to the 207 plants from 63 natural populations, 23 specimens deposited in KYO and OSA were used for morphological analysis to compensate for data scarcity in several regions. Voucher specimens were deposited in the herbarium of Kumamoto University (KUMA).

TABLE 1. Sampling localities of materials analyzed for morphology and molecular variation.

Pop. No.	Locality	No. of plants: Molecular analysis	No. of plants: Morphological analysis	cpDNA haplotype	ITS haplotype	Voucher specimens
1	Hakodate, Hokkaido	1	4	III	6b	*Soejima 020624-1~4*
2	Tsuruoka, Yamagata	1	5	III	3	*Soejima 0209-1,6,12,13,18*
3	Futaba, Fukushima	1	2	III	-	*Soejima 060716-1~2*
4	Tamura, Fukushima	-	2	-	-	*Soejima 060716-3~4*
5	Samekawa, Fukushima	-	2	-	-	*Soejima 060715-3~4*
6	Nasu, Tochigi	1	2	III	6b	*Soejima 060716-5,6*
7	Nikko, Tochigi	1	2	III	3	*Soejima 060715-1,2*
8	Niigata, Niigata	1	5	III	6b	*Soejima 050728-1~5*
9	Uonuma, Niigata	1	6	III	3	*Soejima 050728-9,11~13,15,16*
10	Tateyama, Chiba	1	5	III	3	*Soejima 0207-59~61,64,67*
11	Miyake Is., Tokyo	1	-	III	3	*Kato s.n.1*
12	Kouzu Is., Tokyo	1	1	III	3	*Kato s.n.2*
13	Manazuru, Kanagawa	1	-	III	3	*Soejima-s.n.C1*
14	Fujikawa, Yamanashi	1	3	III	6b	*Soejima 050824-10~12*
15	Chino, Nagano	1	5	III	6b	*Soejima 050824-4~8*
16	Takaoka, Toyama	1	6	III	6	*Soejima 0207-91,96,112,121,123,127*
17	Takayama, Gifu	2	3	IIIb	6	*Soejima 050823-1~3*
18	Shinshiro, Aichi	2	5	IIIb	6b	*Soejima 050824-13,14,16~18*
19	Maibara, Shiga	1	4	III	6b	*Soejima 051105-1~4*
20	Inabe, Mie	-	3	-	-	*Soejima 051105-5~7*
21	Komono, Mie	1	5	III	6b	*051105-8~12*
22	Shima, Mie	1	-	III	8	*Soejima-s.n.C3*
23	Kyoto, Kyoto	2	2	III	6	*Soejima 0209-68,69*
24	Yoshino, Nara	-	2	-	-	*Soejima 051016-5,6*
25	Tenkawa, Nara	1	4	III	8	*Soejima 051016-1~4*
26	Hidaka, Wakayama	1	6	II	1	*Soejima 0205-10,12,14,15,21,24*
27	Himeji, Hyogo	1	4	IV	1	*Soejima 051027-1~4*
28	Shisou, Hyogo	-	4	-	-	*Soejima 051027-5~8*
29	Tottori, Tottori	1	5	IIIb	7	*Soejima 0208-23,25,26,29,32*
30	Un-nan, Shimane	1	3	IV	1	*Soejima 051027-10~12*
31	Yamaguchi, Yamaguchi	-	1	-	-	*Soejima 051028-13*
32	Shuhou, Mine, Yamaguchi	1	2	IV	1	*Soejima 051029-7,8*
33	Mine, Yamaguchi	-	3	-	-	*Soejima 051029-4~6*
34	Shimonoseki, Yamaguchi	1	3	IV	1	*Soejima 051029-1~3*
35	Awaji Is., Hyogo	1	-	III	6	*Naiki 5888*
36	Minami, Tokushima	1	1	IV	7	*Soejima 050914-1*
37	Naka, Tokushima	1	4	I	-	*Soejima 050914-2~5*
38	Kami, Kochi	1	3	I	2	*Soejima 050914-7~9*
39	Tosashimizu, Kochi	1	5	VI	1	*Soejima 0207-4,5,8,9,11*
40	Kumakogen, Ehime	1	1	IV	1	*Soejima 050915-9*
41	Oozu, Ehime	1	1	I	7	*Soejima 050915-8*
42	Kihoku, Ehime	1	5	IV	8	*Soejima 050915-2~6*
43	Hirado, Nagasaki	1	6	IV	1	*Soejima 0209-75,85,90~92,95*
44	Hinokage, Miyazaki	-	2	-	-	*Soejima 060527-1,2*
45	Kadogawa, Miyazaki	1	2	VIb	1	*Soejima 060527-3,4*
46	Takanabe, Miyazaki	1	2	I	1	*Soejima 060527-5,6*
47	Miyazaki, Miyazaki	-	1	-	-	*Soejima 060527-7*

48	Nichinan, Miyazaki	1	3	II	1	*Soejima 060528-1~3*
49	Minami-aso, Kumamoto	1	2	IV	1	*Soejima 060526-1,2*
50	Uto, Kumamoto	-	1	-	-	*Soejima 060529-10*
51	Uki, Kumamoto	1	3	II	1	*Soejima 060529-7~9*
52	Sagara, Kumamoto	1	3	I	1	*Soejima 060529-4~6*
53	Kamiamakusa, Kumamoto	-	2	-	-	*Soejima 0616-5,6*
54	Amakusa, Kumamoto	1	2	II	1c	*Soejima 0614-1,2*
55	Amakusa, Kumamoto	-	2	-	-	*Soejima 0614-3,4*
56	Akune, Kagoshima	1	3	II	1	*Soejima 060529-1~3*
57	Satsuma, Kagoshima	-	1	-	-	*Soejima 060528-9*
58	Kirishima, Kagoshima	1	3	II	1	*Soejima 060528-6~8*
59	Shibushi, Kagoshima	1	2	II	1	*Soejima 060528-4,5*
60	Kuro Is., Kagoshima	1	5	II	1	*Fujii 020961,63~66*
61	Yaku Is., Kagoshima	1	-	II	1c	*107-1-10*
62	Amami, Kagoshima	1	5	V	-	*Soejima 0203-135,137,140,141,144*
63	Tokunoshima, Kagoshima	1	5	II	-	*Soejima 0203-226,228,229,232,235*
64	Kunigami, Okinawa	1	6	VIb	1	*Soejima 0203-52,61,65,66,69,75*
65	Yomitan, Okinawa	1	5	Vb	5	*Soejima 0203-1,2,4~6*
66	Ishigaki, Okinawa	1	6	VII	4	*Soejima 0206-211,214,231,235,239,240*
67	Iriomote, Okinawa	1	5	VII	4	*Soejima 0206-44,47,48,70,75*
68	Pijyarin, Cheju Is.	1	1	IIb	1b	*Soejima 1021*
	Total *	55 (58)	63 (207)	55 (58)	51 (53)	

*: Number of populations and number of individuals in parentheses

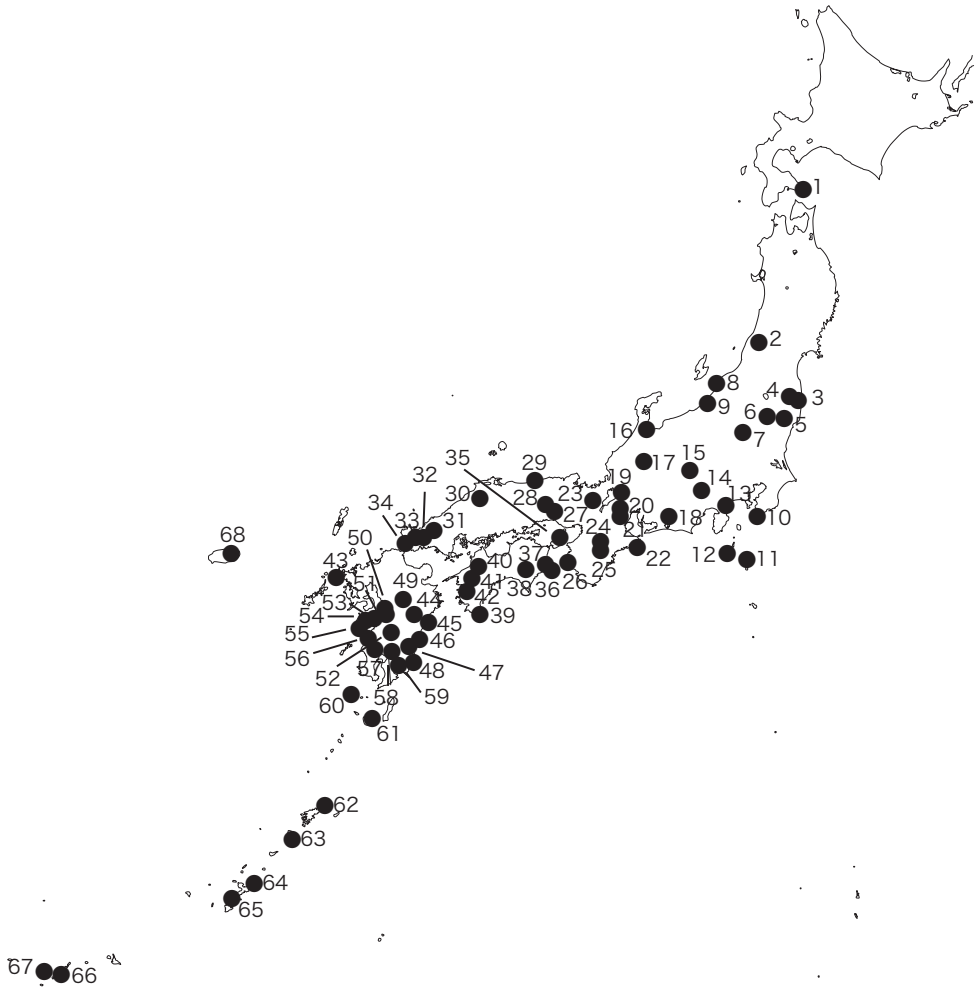

FIG. 1. Locations of populations sampled in this study.

TABLE 2. Designations, sequences, and literature references for the oligonucleotide primers.

Region	Annealing temperature	Primer	Sequence	Reference	Amplicon length
trnH–psbA	52ºC	trnH	ACTGCCTTGATCCACTTGGC	Hamilton 1999	284–369
		psbA	CGAAGCTCCATCTACAAATGG		
trnV–trnM	50ºC	trnV	TGTAAACGAGTTGCTCTACC	Nishizawa & Watano 2000	200
		trnM	CTAACCACTGAGTTAAGTAG		
trnG–trnfM	50ºC	trnG	TCTCTTTGCCAAGGAGAAGA	Nishizawa & Watano 2000	196–234
		trnfM	ATAACCTTGAGGTCACGGGT		
psbC–trnS	54ºC	psbC	TGAACCTGTTCTTTCCATGA	Nishizawa & Watano 2000	261–262
		trnS	GAACTATCGAGGGTTCGAAT		
rps16 intron	50ºC	rpsF	GTGGTAGAAAGCAACGTGCGACTT	Oxelman *et al*. 1997	874–877
		rpsR2	TCGGGATCGAACATCAATTGCAAC		
ITS1	52ºC	ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al*. 1990	296
		ITS2	GCTGCGTTCTTCATCGATGC		
ITS2	50ºC	ITS3	GCATCGATGAAGAACGCAGC	White *et al*. 1990	370–388
		ITS4	TCCTCCGCTTATTGATATGC		

DNA extraction, amplification, and sequencing

Fresh leaf samples were collected and dried in silica gel for DNA extraction. Genomic DNA was extracted following the methods of Doyle & Doyle (1987), with minor changes.

We amplified five noncoding regions of chloroplast DNA (cpDNA), i.e., *psbC–trnS*, *trnV–trnM*, *trnG–trnfM*, *trnH–psbA* intergenic spacers, and *rps*16 intron, and the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) using the primers specified in Table 2. PCR was conducted as follows: 95°C for 2 min; 30 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min (see Table 2 for annealing temperatures), and extension at 72°C for 2 min; and final extension at 72°C for 5 min. Amplified products were purified using 20% polyethylene glycol and 2.5 M NaCl and labeled with a BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The fluorescently labeled samples were run on an ABI PRISM 3100 automated sequencer. Each fragment was directly sequenced from both directions without cloning.

DNA sequence alignment and phylogeny analysis

DNA sequences were aligned with the program CLUSTALX v.1.81 (Thompson *et al*. 1997) using the default penalty settings. The alignment was edited manually using the program Se-Al v.2.0 (Rambaut 1996). Gaps were used as informative characters in defining haplotypes of cpDNA and ITS (Tables 3 and 4). Genetic diversity was assessed for cpDNA and ITS using haplotype diversity H_D and nucleotide diversity π (Nei 1987). Nucleotide diversity was calculated by DnaSP v5.10.1 (Librado & Rozas 2009). We applied the median-joining (MJ) network method (Bandelt et al. 1999) to infer relationships among haplotypes using Network 4.5.1.0 (Fluxus Technology Ltd. at www.fluxus-engineering.com).

Examination of leaf morphological variation

Leaf morphological characters were measured for 230 plants using a mature leaf located at the second node from the branch apex. We measured the following characters: 1, leaf length; 2, leaf length of lower region; 3, leaf length of apical region; 4, leaf width; 5, petiole length; 6, leaf thickness; and 7, leaf area (Fig. 2). Leaf area was calculated by NIH Image 1.62 (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/). Many of the specimens used for measurements bore neither flowers nor fruits. We therefore did not determine the morphological characteristics of the reproductive organs. For the 7 characters, coefficients of pairwise Pearson product-moment correlation were calculated.

Results

We determined the sequences of five cpDNA regions for 58 plants representing 55 populations, and sequences of nrDNA ITS regions for 53

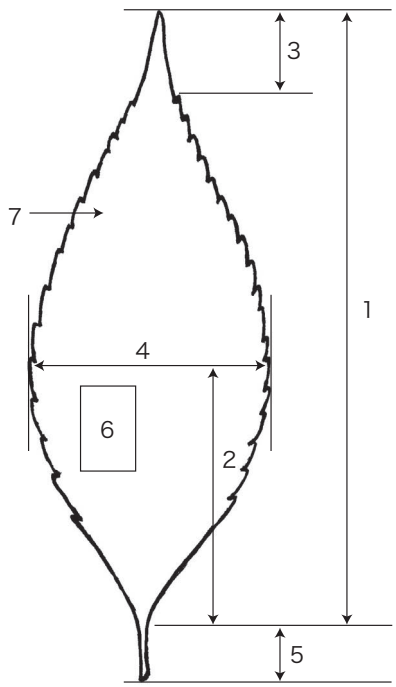

FIG. 2. Drawing of a leaf of *Callicarpa japonica*. 1, leaf length; 2, leaf length of lower region; 3, leaf length of apical region; 4, leaf width; 5, petiole length; 6, leaf thickness; 7, leaf area.

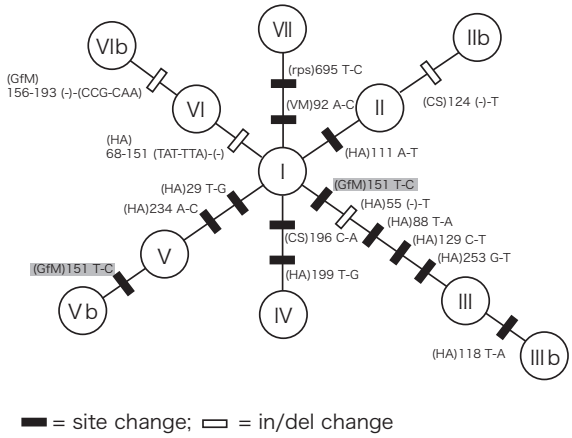

FIG. 3. Network relationships among chloroplast haplotypes obtained using the MJ network method. Solid bars indicate site changes; open bars indicate insertion and/or deletion changes. Site changes considered to be convergence are shaded gray.

plants from 51 populations. All of the obtained sequence data was deposited in DDBJ under accession numbers AB751662–AB752089. The length of the nucleotide sequences of the five cpDNA regions, ITS1, and ITS 2 are shown in Table 2. No heterozygotic sequence patterns were found for the ITS1 and ITS2 regions. Based on substitutions and insertion/deletion changes, 11 haplotypes for cpDNA and 11 haplotypes for ITS were recognized (Tables 3 and 4). Natural hybridization and reciprocal introgression between *Callicarpa japonica* and *C. mollis* Sieb. et Zucc. have been reported in central Japan (Tsukaya *et al*. 2003). We confirmed that the samples used in our study were not influenced by hybridization by comparing our ITS sequences with those of *C. mollis* (AB099648).

Figure 3 shows the relationships among the cpDNA haplotypes in the MJ network. The cpDNA haplotypes were named as I, II, IIb, III, IIIb, IV, V, Vb, VI, VIb, and VII. A terminal node connected to its respective internal node by only 1 mutation was indicated by adding an alphabetical suffix. The geographic distribution of the haplotypes is shown in Fig. 4. Haplotypes III and IIIb were restricted to the eastern region; no other haplotypes were found in that region. The other nine haplotypes were found only in the western region. The boundary between the distribution range of haplotype III (and IIIb) and the other nine haplotypes was in the Kinki district.

Figure 5 shows the relationships among the ITS haplotypes in the MJ network. The ITS haplotypes were designated 1, 1b, 1c, 2, 3, 4, 5, 6, 6b, 7, and 8. A terminal node connected to its respective internal node by only 1 mutation was indicated by adding an alphabetical suffix. The geographic distribution of the ITS haplotypes is shown in Fig. 6. Haplotypes 3, 6, 6b and 8 were mostly found in the eastern region, but haplotype 8 was exceptionally found in one population in the western region. No other haplotypes were found in eastern Japan, while the other haplotypes, i.e., 1, 1b, 1c, 2, 4, 5, and 7, were found only in the western region. The boundary dividing the populations into western and eastern groups was

TABLE 3. Site changes and haplotypes of plastid DNA regions.

	Primers and positions																								
	CS		VM	GfM				HA															rps		frequency (Total 55)
	1	1		1	1		1					1		1	1	1	1	2	2	6				
	2	9	9	5	5	–	9	2	5	6	–	5	8	1	1	2	9	3	5	9				
Haplotype	4	6	2	1	6		3	9	5	8		1	8	1	8	9	9	4	3	5				
Ⅰ	–	C	A	T	–			T	–	β			T	A	T	C	T	A	G	T				0.09 (5)
Ⅱ	•	•	•	•	•			•	•	•			•	T	•	•	•	•	•	•				0.18 (10)
Ⅱb	T	•	•	•	•			•	•	•			•	T	•	•	•	•	•	•				0.02 (1)
Ⅲ	•	•	•	C	•			•	T	•			A	•	•	T	•	•	T	•				0.36 (20)
Ⅲb	•	•	•	C	•			•	T	•			A	•	A	T	•	•	T	•				0.05 (3)
Ⅳ	•	A	•	•	•			•	•	•			•	•	•	•	G	•	•	•				0.16 (9)
Ⅴ	•	•	•	•	•			G	•	•			•	•	•	•	•	C	•	•				0.02 (1)
Ⅴb	•	•	•	C	•			G	•	•			•	•	•	•	•	C	•	•				0.02 (1)
Ⅵ	•	•	•	•	•			•	•	–			•	•	•	•	•	•	•	•				0.02 (1)
Ⅵb	•	•	•	•	α			•	•	–			•	•	•	•	•	•	•	•				0.04 (2)
Ⅶ	•	•	C	•	•			•	•	•			•	•	•	•	•	•	•	C				0.04 (2)

•:Same as genotype I, –:absence
CS = *psbC–trnS*, VM = *trnV–trnM*, GfM = *trnG–trnfM*, HA = *trnH–psbA*, rps = *rps*16 intron
α : CCGAAAAGGGGTACTAGTTAACAGAGCCAAACCTCCAA (38 bp)
β : TATTATTTTATTTATTATTATTATTTAAATTTAAATATTTAAATTATTTACGAATTCGATTTTTCAATTTTTA (73 bp)
Observed numbers are shown in parentheses.

TABLE 4. Site changes and haplotypes of ITS1 and ITS2.

	Primers and positions																												
	ITS1											ITS2																	frequency (Total 51)
					1	1	1	2	2	2	2	1	1	1		1	1	1	2	2	2	3	3	3			3	3	
	7	8	9	9	1	2	9	3	3	3	5	4	6	7	–	8	8	9	1	5	7	0	1	2	–		3	4	
Haplotype	2	2	0	6	8	6	2	0	1	3	0	6	7	7		3	7	4	6	0	8	2	3	0			7	0	
1	G	C	C	G	T	G	A	G	C	C	T	C	C		-		C	T	G	C	C	C	A	TTGCACGCACGCGTGCAC				T	0.37 (19)
2	•	•	•	•	•	•	•	C	•	G	•	•	•		•		•	•	•	•	•	•	•		•			•	0.02 (1)
1c	•	•	•	C	•	•	•	•	•	•	•	•	•		•		•	•	•	•	•	•	•		•			•	0.04 (2)
1b	•	•	•	•	•	•	•	•	T	•	•	•	•		•		•	•	•	•	•	•	•		•			•	0.04 (2)
3	C	•	T	•	•	•	•	•	•	•	•	A	•		GCCTCCG		T	•	•	•	•	•	•		•			C	0.14 (7)
4	C	G	•	•	•	•	•	•	•	•	•	•	T		GCCTCCG		•	C	•	•	•	A	-	--------A---------				C	0.04 (2)
5	C	G	•	•	•	•	•	•	•	•	C	•	T		GCCTCCG		•	C	•	•	•	G	-	--------A---------				C	0.02 (1)
6	C	•	•	•	•	•	•	•	•	•	•	•	•		•		•	C	A	•	T	A	-	TTGCACGC----GTGCAC				C	0.08 (4)
6b	C	•	•	•	•	•	•	•	•	•	•	•	•		•		•	C	A	T	T	A	-	TTGCACGC----GTGCAC				C	0.16 (8)
7	C	•	•	•	A	•	G	•	•	•	•	•	•		•		•	•	•	•	•	•	•		•			•	0.04 (2)
8	C	•	•	•	•	•	•	•	•	•	•	A	•		•		•	C	•	•	•	•	•		•			C	0.06 (3)

•: Same as haplotype 1, -: absence
Observed numbers are shown in parentheses.

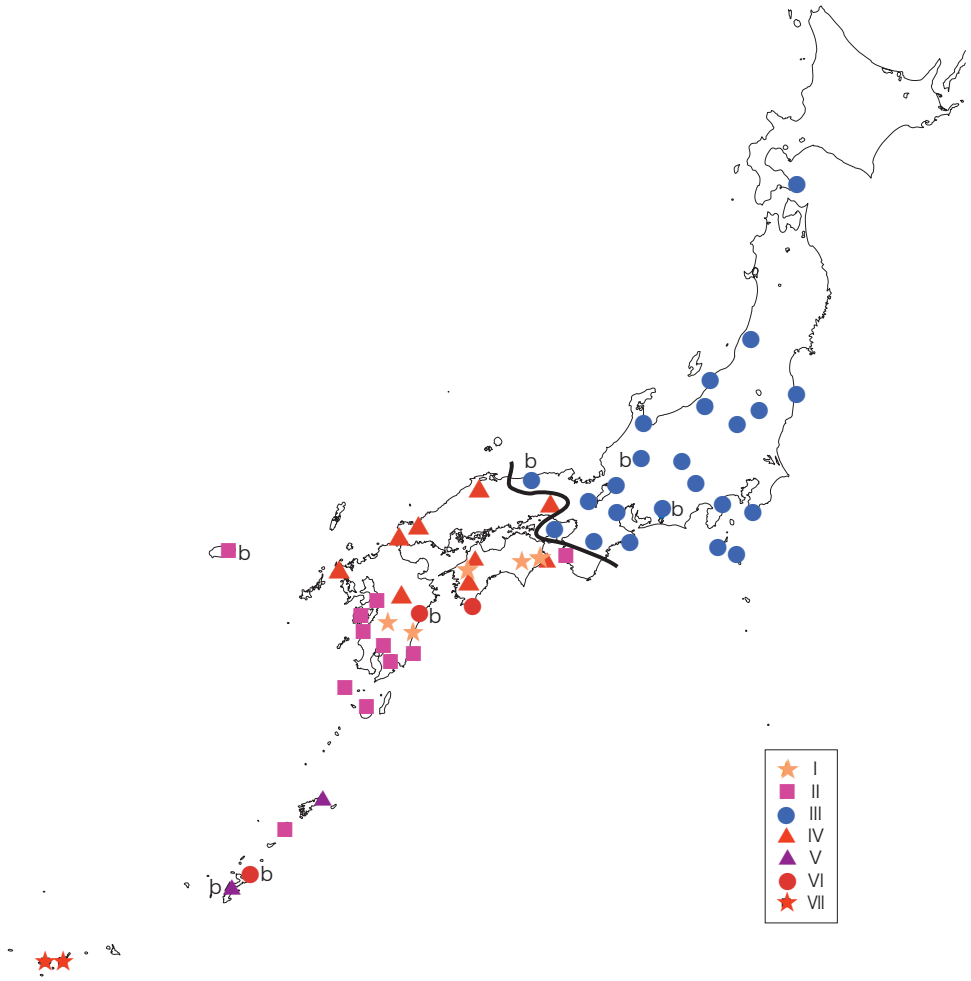

FIG. 4. Geographic distribution of chloroplast haplotypes. Symbols of haplotypes of IIb, IIIb, Vb and VIb are shown by adding "b" to the symbols of haplotypes II (magenta square), III (blue circle), V (purple triangle) and VI (red circle).

identical to that for cpDNA, except for population 29 (Tottori Pref.), which contained an eastern cpDNA haplotype (IIIb) and a western ITS haplotype (7). Additionally, population 42 (Ehime Pref.) on the west side of the boundary line contained a western cpDNA haplotype (IV) and an eastern ITS haplotype (8). Based on the geographical distribution pattern of both the cpDNA and the ITS haplotypes, except for these two populations, the populations examined were geographically divided into eastern (eastern cpDNA and eastern ITS haplotypes: EE) and western (western cpDNA and western ITS haplotypes: WW) population groups. Table 5 shows the frequencies of the cpDNA and the ITS haplotypes and the genetic diversity indices (H_D and π) in the western and eastern population groups. Populations 29 and 42 were excluded from the calculations because it was difficult to classify them into either. For chloroplast DNA, haplotype diversity and nucleotide diversity was considerably higher in western Japan (H_D = 0.820, $\pi = 1.58 \times 10^{-3}$) than in eastern Japan (H_D = 0.172, $\pi = 0.05 \times 10^{-3}$). For the ITS region, however, both diversity indices were slightly higher in eastern Japan: H_D = 0.537 and 0.736, $\pi = 3.73 \times 10^{-3}$ and 7.33×10^{-3} in western and eastern Japan, respectively.

Means and standard deviations of the seven characters of leaf morphology are shown for each population in Appendix 1. Among the seven

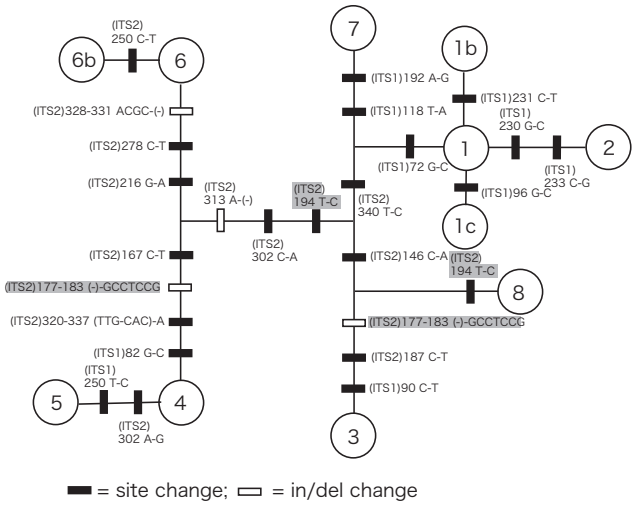

FIG. 5. Network relationships among chloroplast haplotypes obtained using the MJ network method. Solid bars indicate site changes; open bars indicate insertion and/or deletion changes. Site changes considered to be convergence are shaded gray.

characters, we detected high correlations between leaf length and leaf width (correlation coefficient r = 0.86), leaf length and leaf length of lower region (r = 0.94), leaf length and petiole length (r = 0.73), leaf area and leaf width (r = 0.96), and leaf area and leaf length (r = 0.92). Large and thick leaves were characteristic of var. *luxurians*, but the correlation coefficient between leaf area and leaf thickness was quite low (r = 0.20).

Figure 7 shows the frequency distribution of leaf area and leaf thickness for the 230 individual plants measured. The frequencies of individuals belonging to the EE populations (65) and individuals belonging to the WW populations (89) are shown separately. Both leaf area and leaf thickness were not normally distributed, but rather skewed to the left. The mean value of the leaf

FIG. 6. Geographic distribution of ITS haplotypes. Symbols of haplotypes of 1b, 1c and 6b are shown by adding alphabetical suffix to symbols of haplotypes 1 (red circle) and 6 (blue circle). Red circle with suffix of c* denotes the population having with both haplotypes 1 and 1c.

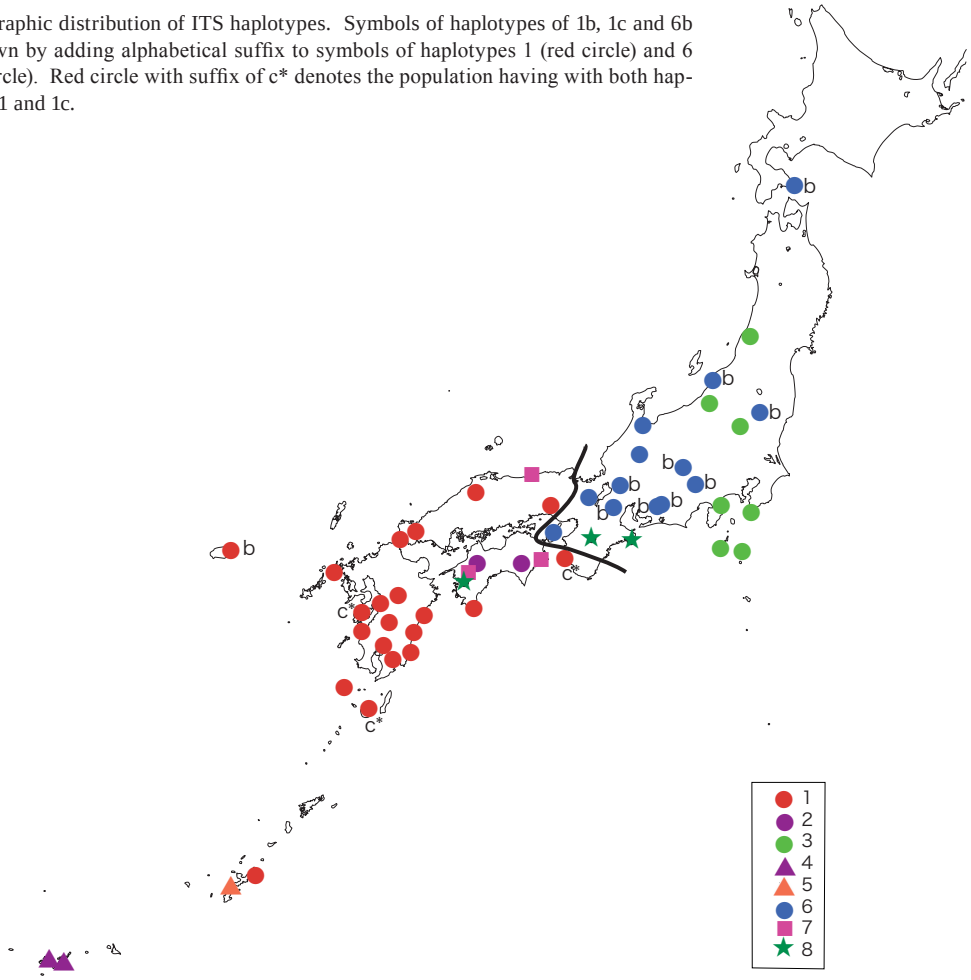

TABLE 5. Frequency of haplotypes and genetic diversity

cpDNA haplotype																	
	n	I	II	II b	III	III b	IV	V	V b	VI	VI b	VII	H_D	π
Western population group	31	0.16 (5)	0.32(10)	0.03 (1)	0.00	0.00	0.26 (8)	0.03 (1)	0.03 (1)	0.03 (1)	0.06 (2)	0.06 (2)	0.820	1.58×10^{-3}
Eastern population group	22	0.00	0.00	0.00	0.91(20)	0.09 (2)	0.00	0.00	0.00	0.00	0.00	0.00	0.172	0.05×10^{-3}

ITS haplotype														
	n	1	1b	1c	2	3	4	5	6	6b	7	8	H_D	π
Western population group	28	0.68(19)	0.04 (1)	0.07 (2)	0.07 (2)	0.00	0.07 (2)	0.04 (1)	0.00	0.00	0.04 (1)	0.00	0.537	3.73×10^{-3}
Eastern population group	21	0.00	0.00	0.00	0.00	0.33 (7)	0.00	0.00	0.19 (4)	0.38 (8)	0.00	0.10 (2)	0.736	7.33×10^{-3}

n = number of total individuals, H_D = haplotype diversity, π = nucleotide diversity
Observed numbers are shown in parentheses.

area was 20.4 ± 12.7 cm^2 for the EE populations and 21.5 ± 14.4 cm^2 for the WW populations. The mean value of leaf thicknesses was 4.6 ± 1.9 μm for the EE populations and 5.8 ± 2.2 μm for the WW populations. Figures 8 and 9 show geographical variation in leaf area and thickness according to the average of each population. Measurements obtained from herbarium specimens are included in these figures. As shown, large and/or thick leaves tended to occur in coastal regions.

Discussion

Genetic structure and phylogeography of Callicarpa japonica *in Japan*

For cpDNA, a genetic border between the populations of eastern and western Japan was recognized in the Kinki district (a tentative line is shown in Fig. 4). In eastern Japan, only haplotypes III and IIIb occurred, while the other nine haplotypes were distributed in western Japan. According to the network of cpDNA haplotypes, the haplotypes appear to radiate from haplotype I (Fig. 3). Haplotypes III and IIIb, found only in eastern Japan, were genetically most distant from haplotype I. This phylogeographic distribution pattern of the cpDNA haplotypes resembles those in other Japanese temperate deciduous trees, such as *Fagus* (Okaura & Harada 2002, Hiraoka & Tomaru 2009) and *Carpinus, Euonymus, Magnolia*, and *Padus* (Iwasaki *et al*. 2010, 2012). Although their phylogeographic breaks are not completely matched, they all show genetic differentiation between eastern and western parts of Japan.

The phylogeographic break observed between populations of eastern and western Japan was also found for the ITS haplotypes in the Kinki district (a tentative line is shown in Fig. 6), but the border was not quite the same as for the cpDNA, i.e., population 29 (Tottori Pref.) and population 42 (Ehime Pref.). Additionally, the phylogeographic distribution pattern of the ITS haplotypes was more complicated than in the cpDNA haplotypes. Because the three eastern haplotypes (3, 6, and 8) do not appear to be closely related to each others (Fig. 5), it is possible that the divergence of

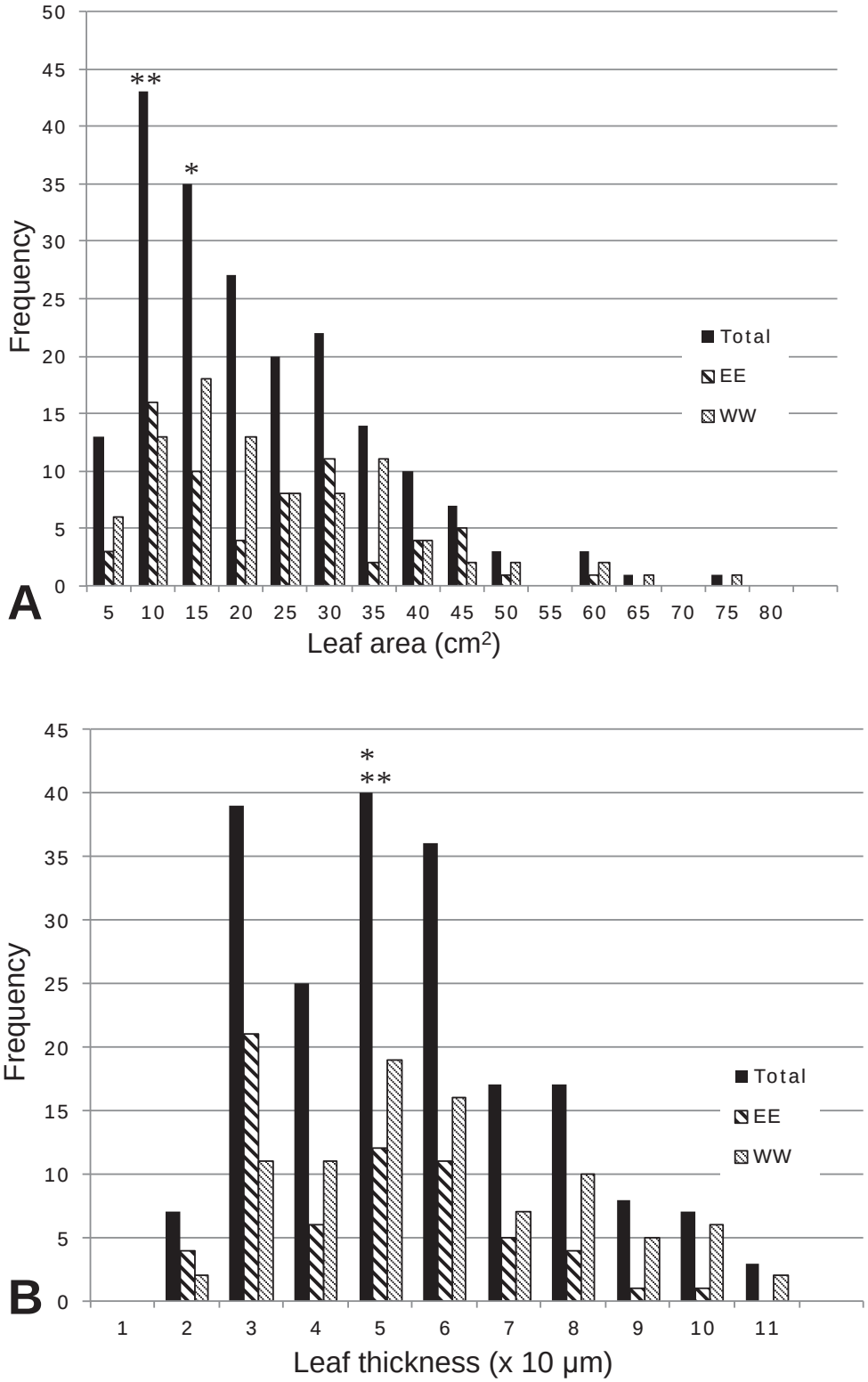

FIG. 7. Frequency distributions of A: leaf area, and B: leaf thickness. Striped bars indicate plants in populations with eastern cpDNA and eastern ITS haplotypes (EE); dotted bars indicate plants in the populations with western cpDNA and western ITS haplotypes (WW). Solid bars indicate all individuals, including those from herbarium specimens, used in morphological analyses. Frequencies are shown as individual numbers. * and ** indicate grade of EW and WE plants, respectively.

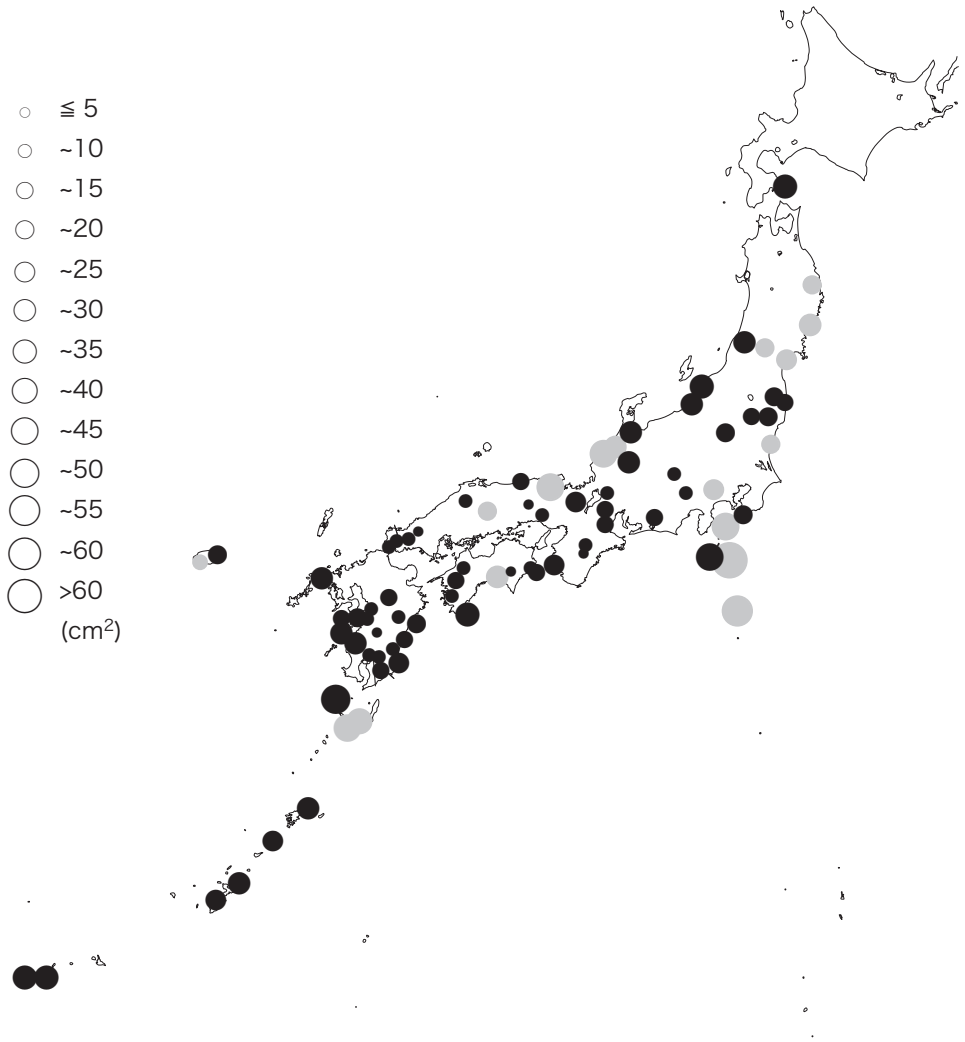

FIG. 8. Variation in leaf area is shown by size of circles. Solid circles indicate populations used for DNA analyses in this study; gray circles indicate individuals measured from herbarium specimens.

the ITS haplotypes occurred in their ancestral populations before the western and eastern population groups separated.

Based on the genetic structure of the cpDNA and ITS, the populations of *Callicarpa japonica* were divided into western (WW) and eastern (EE) population groups separated at the Kinki district. These results coincide with other plants shown in previous previous studies (Okaura & Harada 2002, Hiraoka & Tomaru 2009, Iwasaki *et al.* 2010, 2012), which suggest isolation between western and eastern plant populations during the Last Glacial Maximum. It seems that the western and eastern populations of *C. japonica* may have expanded their distribution ranges from their respective refugia independently after the last glacial period, and come into contact near Kinki district. Although the genetic diversity of the ITS region was slightly higher in the eastern populations (Table 5), the numbers of ITS haplotypes is higher in the western groups as is the case with the cpDNA haplotypes. It may suggest that refugia in western Japan were greater in number and/or size than those in eastern Japan.

Compared with ITS, the genetic structure of the cpDNA is expected to be more sensitive to

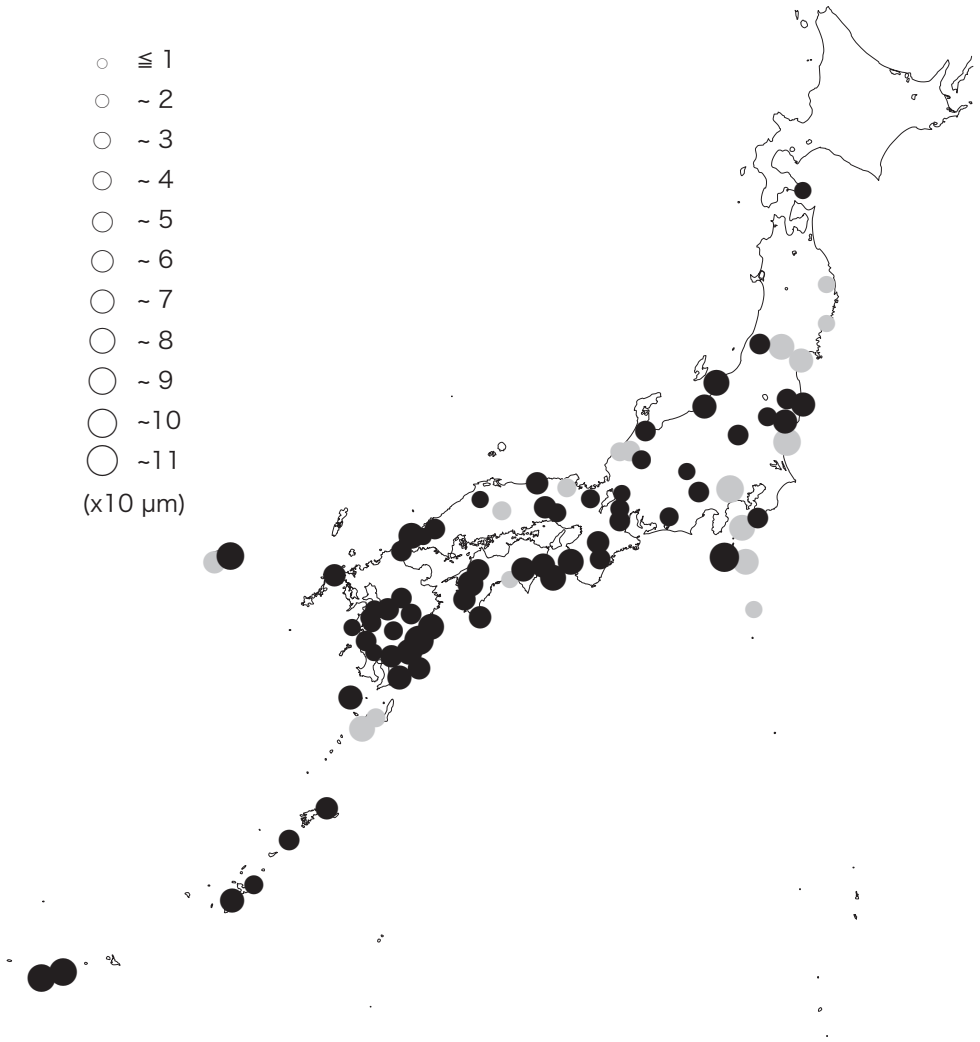

FIG. 9. Variation in leaf thickness is shown by size of circles. Solid circles indicate populations used for DNA analyses in this study; gray circles indicate individuals measured from herbarium specimens.

bottlenecks and genetic drift because of its haploid nature. If so, the genetic structure of the cpDNA could well reflect historical changes in distribution under the influence of climatic fluctuations during and after the last glacial period. It is possible that the present genetic structure of ITS retains more information of the genetic structure before the putative population reduction during the last glacial period.

Relationship between morphological variation and genetic structure

The range of morphological variation within *Callicarpa japonica* is relatively large, resulting in two varieties, *japonica* and *luxurians*, based on differences in leaf area and leaf thickness being recognized in Japan (Kitamura & Murata 1971, Murata 1989, Yamazaki 1993). The large variation in leaf area and leaf thickness was confirmed in this study, but our data do not support recognition of infraspecific taxa. Leaf area correlated with leaf width and leaf length, but not with leaf

thickness. The data indicate that larger leaves are not necessarily thicker, and vice versa in *C. japonica*. Moreover, values for leaf area and leaf thickness varied continuously from one extreme the other (Fig. 7), making it difficult to distinguish varieties based on these characteristics. Compared with the genetic structure, the values for leaf area and thickness were slightly greater in the WW populations than in the EE populations, but the ranges in variation mostly overlapped (Fig. 7). From these data, morphological differences do not appear to correlate with genetic differences in the cpDNA and rDNA ITS regions. Differences between vars. *japonica* and *luxurians* are therefore not supported phylogenetically. It should be noted that the plants from Cheju Island (Korea), the type locality of var. *luxurians*, observed in this study (population 68), exhibited the western cpDNA and western ITS haplotypes (Figs. 4 and 6), and produced relatively thick, small leaves (Figs. 8 and 9).

Our data also demonstrated that large and/or thick leaves tend to occur in coastal regions (Figs. 8 and 9). The upper surface of large, thick leaves is usually lustrous (for example, in populations 8, 11, 39, 60, 66, 67, personal observation), which may be a response to strong sunlight and aridity. These characteristics may imply adaptation to a coastal environment. Plants with large, thick leaves in the western and eastern regions may be due to convergence through adaptation to similar environmental conditions.

Many deciduous trees along the coast of the Sea of Japan have larger leaves than their conspecific populations on the Pacific coast (Hotta 1974, Hagiwara 1977). Some populations with large and/or thick leaves, as occur in the coastal region of the Sea of Japan in this study (such as populations 1, 2, 17), may be such examples. Large and/or thick leaves appear to be among the characteristics of adaptation to coastal habitats in the eastern and western population groups. It should be noted, however, that the distribution range of *Callicarpa japonica* extends to Taiwan, Korea and southern China (Yamazaki 1993, Chen *et al.* 1998). To fully elucidate the evolutionary history and infraspecific taxonomy of *C. japonica*, populations from these other regions must be investigated.

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APPENDIX 1. Leaf measurements of *Callicarpa japonica* (mean and standard deviation).

Pop. No.	Locality	N	leaf length (cm)	l.l. lower part (cm)	l.l. apical part (cm)	leaf width (cm)	petiole length (cm)	leaf thickness (x 10 μm)	leaf area (cm2)
1	Hakodate, Hokkaido	4	11.61 ± 1.36	6.01 ± 1.34	1.15 ± 0.21	4.27 ± 0.88	0.45 ± 0.12	3.00 ± 0.00	30.46 ± 7.73
2	Tsuruoka, Yamagata	5	10.99 ± 1.75	5.19 ± 1.14	1.58 ± 0.40	4.55 ± 0.78	0.76 ± 0.18	4.50 ± 1.12	28.67 ±13.44
3	Futaba, Fukushima	2	9.42 ± 1.86	4.37 ± 0.22	1.89 ± 0.59	2.65 ± 0.39	0.48 ± 0.11	7.00 ± 1.41	13.85 ± 4.83
4	Tamura, Fukushima	2	11.16 ± 2.81	5.48 ± 1.20	2.29 ± 1.20	3.17 ± 0.23	0.59 ± 0.16	4.50 ± 0.71	19.68 ± 5.90
5	Samekawa, Fukushima	2	9.71 ± 1.49	4.52 ± 0.82	1.58 ±0.53	3.12 ± 0.54	0.44 ± 0.03	6.25 ± 3.18	16.86 ± 0.64
6	Nasu, Tochigi	2	8.90 ± 0.52	3.77 ± 0.33	1.57 ± 0.49	2.71 ± 0.11	0.49 ± 0.12	3.75 ± 1.06	13.95 ± 0.42
7	Nikko, Tochigi	2	9.34 ± 2.17	5.01 ± 1.32	1.29 ± 0.81	3.18 ± 0.31	0.37 ± 0.01	4.75 ± 0.35	17.63 ± 5.26
8	Niigata, Niigata	5	12.09 ± 1.48	5.40 ± 0.92	1.76 ± 0.49	4.89 ± 1.23	0.53 ± 0.14	7.20 ± 0.76	34.86 ± 12.95
9	Uonuma, Niigata	6	11.12 ± 1.87	4.92 ± 1.05	1.93 ± 0.27	4.03 ± 1.02	0.71 ± 0.20	6.92 ± 1.66	26.46 ± 9.75
10	Tateyama, Chiba	5	9.37 ± 3.59	3.78 ± 1.49	1.67 ± 0.56	3.79 ± 1.37	0.46 ± 0.16	4.10 ± 2.01	22.88 ± 15.40
12	Kouzu Is., Tokyo	1	11.21	5.69	0.77	6.17	1.31	10.00	42.46
14	Fujikawa, Yamanashi	3	6.55 ± 1.08	2.60 ± 0.55	1.17 ± 0.15	2.39 ± 0.55	0.39 ± 0.08	4.50 ± 1.32	9.25 ± 3.59
15	Chino, Nagano	5	5.97 ± 1.04	2.75 ± 0.51	1.03 ± 0.39	2.08 ± 0.31	0.30 ± 0.07	3.00 ± 0.79	7.74 ± 1.85
16	Takaoka, Toyama	6	11.28 ± 1.73	5.39 ± 0.99	1.89 ± 0.72	4.29 ± 1.32	0.56 ± 0.11	5.00 ± 1.55	27.44 ± 10.18
17	Takayama, Gifu	3	11.76 ± 2.88	5.12 ± 0.74	2.23 ± 0.68	3.50 ± 0.86	0.45 ± 0.17	3.83 ± 1.89	25.44 ± 10.23
18	Shinshiro, Aichi	5	7.93 ± 2.18	3.59 ± 0.87	1.64 ± 0.83	2.92 ± 0.75	0.37 ± 0.12	3.60 ± 0.89	14.03 ± 7.22
19	Maibara, Shiga	4	6.68 ± 1.16	3.13 ± 0.74	1.02 ± 0.36	2.19 ± 0.18	0.30 ± 0.03	2.50 ± 0.58	8.67 ± 1.85
20	Inabe, Mie	3	8.18 ± 0.54	3.31 ± 0.57	1.24 ± 0.21	2.87 ± 0.36	0.26 ± 0.01	3.17 ± 0.76	13.88 ± 2.42
21	Komono, Mie	5	7.85 ± 1.18	3.45 ± 0.75	1.50 ± 0.31	2.76 ± 0.83	0.46 ± 0.05	4.50 ± 1.00	12.51 ± 5.83
23	Kyoto, Kyoto	2	9.99 ± 0.29	5.13 ± 0.15	1.17 ± 0.23	3.81 ± 0.06	0.51 ± 0.05	3.50 ± 0.71	21.98 ± 0.91
24	Yoshino, Nara	2	5.48 ± 0.04	2.13 ± 0.12	1.20 ± 0.25	1.69 ± 0.11	0.23 ± 0.00	5.50 ± 1.41	5.26 ± 0.07
25	Tenkawa, Nara	4	5.26 ± 1.36	2.18 ± 0.47	1.04 ± 0.51	1.52 ± 0.22	0.22 ± 0.06	4.88 ± 0.48	4.91 ± 1.78
26	Hidaka, Wakayama	6	10.04 ± 2.09	4.43 ± 0.83	1.56 ± 0.83	4.43 ± 0.59	0.83 ± 0.24	7.08 ± 1.74	26.34 ± 7.08
27	Himeji, Hyogo	4	5.76 ± 1.81	2.59 ± 0.97	0.77 ± 0.48	1.92 ± 0.43	0.34 ± 0.18	3.75 ± 0.50	7.05 ± 3.40
28	Shisou, Hyogo	4	5.05 ± 1.16	2.15 ± 0.40	0.86 ± 0.40	1.44 ± 0.13	0.19 ± 0.05	5.50 ± 2.52	4.45 ± 1.36
29	Tottori, Tottori	5	7.26 ± 1.84	3.43 ± 0.55	0.74 ± 0.25	2.99 ± 0.92	0.41 ± 0.11	5.20 ± 1.10	13.37 ± 7.48
30	Un-nan, Shimane	3	7.10 ± 1.68	3.54 ± 0.76	0.89 ± 0.34	2.44 ± 0.83	0.27 ± 0.10	2.67 ± 0.58	10.07 ± 5.15
31	Yamaguchi, Yamaguchi	1	5.07	1.96	0.93	1.39	0.20	5.00	3.99
32	Shuhou, Mine, Yamaguchi	2	6.47 ± 1.42	2.55 ± 0.03	2.87 ± 0.47	0.89 ± 0.82	0.28 ± 0.01	3.00 ± 0.00	9.57 ± 1.51
33	Mine, Yamaguchi	3	5.90 ± 0.50	1.80 ± 0.20	2.65 ± 0.14	1.24 ± 0.11	0.22 ± 0.03	7.33 ± 2.75	5.94 ± 1.02
34	Shimonoseki, Yamaguchi	3	6.23 ± 1.45	2.59 ± 0.59	2.88 ± 0.81	1.15 ± 0.28	0.29 ± 0.03	4.83 ± 0.29	9.58 ± 3.80
36	Minami, Tokushima	1	6.60	2.99	3.60	1.10	0.48 ± 0.00	8.00 ± 0.00	12.05 ± 0.00
37	Naka, Tokushima	4	5.88 ± 0.70	2.23 ± 0.44	2.44 ± 0.58	0.95 ± 0.15	0.30 ± 0.08	6.75 ± 0.50	7.98 ± 1.93
38	Kami, Kochi	3	5.22 ± 0.10	1.68 ± 0.17	2.30 ± 0.19	1.01 ± 0.28	0.26 ± 0.03	7.00 ± 1.00	4.97 ± 0.80
39	Tosashimizu, Kochi	5	12.31 ± 2.04	4.48 ± 0.57	5.58 ± 0.90	2.17 ± 0.62	1.39 ± 0.46	5.20 ± 1.79	33.05 ± 8.77
40	Kumakogen, Ehime	1	5.90	2.39	2.63	0.63	0.15	5.50	8.56
41	Oozu, Ehime	1	7.02	2.92	2.68	1.27	0.27	7.50	11.70
42	Kihoku, Ehime	5	4.72 ± 1.12	1.87 ± 0.38	2.16 ± 0.54	0.80 ± 0.41	0.25 ± 0.07	5.40 ± 0.89	5.22 ± 1.80
43	Hirado, Nagasaki	6	10.08 ± 2.55	4.55 ± 0.81	4.75 ± 0.82	1.12 ± 0.65	0.85 ± 0.32	5.50 ± 1.95	28.02 ± 10.88
44	Hinokage, Miyazaki	2	6.34 ± 1.87	2.49 ± 0.13	2.49 ± 0.47	1.13 ± 0.59	0.24 ± 0.08	4.50 ± 0.71	9.67 ± 3.25
45	Kadogawa, Miyazaki	2	7.84 ± 0.71	3.24 ± 0.08	3.91 ± 0.78	1.13 ± 0.21	0.46 ± 0.20	7.75 ± 0.35	15.58 ± 1.82
46	Takanabe, Miyazaki	2	7.12 ± 0.35	2.83 ± 0.18	3.22 ± 0.38	1.22 ± 0.56	0.41 ± 0.13	9.50 ± 0.71	11.28 ± 1.22
47	Miyazaki, Miyazaki	1	6.11	2.21	2.99	1.02	0.29	8.00	8.31
48	Nichinan, Miyazaki	3	8.81 ± 0.99	3.77 ± 0.36	4.24 ± 0.58	1.11 ± 0.40	0.86 ± 0.27	5.67 ± 0.58	20.65 ± 3.35
49	Minami-aso, Kumamoto	2	7.53 ± 0.23	2.79 ± 0.23	3.76 ± 0.50	1.19 ± 0.16	0.33 ± 0.08	5.00 ± 0.00	12.18 ± 1.24
50	Uto, Kumamoto	1	5.01	2.36	2.64	0.58	0.22	6.00	7.35
51	Uki, Kumamoto	3	6.47 ± 1.41	2.79 ± 0.61	2.83 ± 0.63	1.07 ± 0.50	0.31 ± 0.05	4.00 ± 0.00	10.28 ± 3.00
52	Sagara, Kumamoto	3	4.48 ± 0.52	1.65 ± 0.23	1.78 ± 0.39	0.62 ± 0.39	0.13 ± 0.03	5.00 ± 0.87	4.77 ± 1.02
53	Kamiamakusa, Kumamoto	2	7.67 ± 0.35	3.37 ± 0.18	4.00 ± 0.23	0.47 ± 0.18	0.47 ± 0.03	4.00 ± 0.71	16.75 ± 1.41
54	Amakusa, Kumamoto	2	7.37 ± 1.24	2.87 ± 0.15	3.49 ± 0.31	1.61 ± 0.12	0.29 ± 0.06	4.00 ± 1.41	12.09 ± 2.34
55	Amakusa, Kumamoto	2	9.34 ± 1.97	4.76 ± 1.46	4.51 ± 0.41	0.95 ± 0.18	0.42 ± 0.04	2.25 ± 0.35	26.66 ± 13.72
56	Akune, Kagoshima	3	11.85 ± 0.46	4.32 ± 0.71	5.68 ± 0.32	1.59 ± 0.28	0.78 ± 0.13	4.67 ± 0.58	29.61 ± 6.19
57	Satsuma, Kagoshima	1	5.30	1.75	2.37	0.83	0.10	3.00	5.98
58	Kirishima, Kagoshima	3	5.86 ± 0.95	2.26 ± 0.29	2.39 ± 0.45	1.10 ± 0.45	0.18 ± 0.02	5.83 ± 0.29	7.94 ± 1.70
59	Shibushi, Kagoshima	2	7.17 ± 0.78	2.79 ± 0.23	3.64 ± 0.62	0.38 ± 0.42	0.54 ± 0.05	6.50 ± 0.71	12.88 ± 0.36
60	Kuro Is., Kagoshima	5	14.15 ± 1.78	5.47 ± 1.32	6.75 ± 0.73	1.72 ± 0.76	1.55 ± 0.40	6.20 ± 1.30	48.74 ± 18.44
62	Amami, Kagoshima	5	9.47 ± 1.47	4.38 ± 0.99	4.95 ± 0.79	0.49 ± 0.22	0.90 ± 0.22	5.70 ± 1.10	27.31 ± 9.63
63	Tokunoshima, Kagoshima	5	8.87 ± 1.22	4.19 ± 0.68	4.05 ± 0.72	0.86 ± 0.53	0.72 ± 0.24	4.10 ± 0.96	23.05 ± 4.65
64	Kunigami, Okinawa	6	9.30 ± 2.80	4.29 ± 1.26	4.21 ± 0.89	1.12 ± 0.93	0.81 ± 0.22	3.33 ± 1.40	26.42 ± 15.63
65	Yomitan, Okinawa	5	9.66 ± 2.24	3.93 ± 0.63	4.83 ± 1.46	0.62 ± 0.19	0.88 ± 0.16	6.30 ± 1.72	24.37 ± 4.44
66	Ishigaki, Okinawa	6	10.84 ± 1.44	4.88 ± 1.04	5.61 ± 0.69	1.14 ± 0.50	1.23 ± 0.23	8.83 ± 1.83	33.25 ± 8.72
67	Iriomote, Okinawa	5	11.09 ± 1.81	4.89 ± 1.35	5.75 ± 1.23	0.78 ± 0.27	0.85 ± 0.27	8.90 ± 2.30	34.87 ± 15.60
68	Pijyarin, Cheju Is.	1	9.72	2.81	3.53	2.08	0.37	8.50	15.44
Herbarium specimens in KYO and OSA									
	Akita		9.44	4.69	1.67	4.32	0.53	5.00	24.02
	Akita		10.87	5.01	1.60	4.30	0.54	3.00	27.00
	Mt. Funagata, Yamagata		7.67	3.28	1.56	2.75	0.69	8.00	13.45
	Sendai, Miyagi		8.09	4.75	0.90	4.00	0.63	7.00	18.21
	Shimoina, Iwate		6.56	3.14	0.96	3.18	0.38	3.00	13.45
	Oofunado, Iwate		8.93	4.63	1.57	3.96	0.60	2.50	20.39
	Takaghagi, Ibaraki		8.05	4.23	0.81	2.40	0.53	9.00	14.01
	Takaghagi, Ibaraki		7.17	3.80	0.44	2.61	0.56	8.00	14.01
	Kaga, Ishikawa		11.57	4.50	1.34	5.30	0.51	4.00	36.63
	Komatsu, Ishikawa		11.20	5.27	2.39	4.30	0.48	5.00	25.38
	Komatsu, Ishikawa		8.85	4.46	1.60	3.81	0.67	4.00	16.77
	Mt. Takao, Tokyo		9.36	4.32	1.38	2.93	0.78	9.00	15.88
	Miyake Is., Tokyo		19.60	8.96	2.12	8.97	2.20	8.00	69.77
	Ooshima Is., Tokyo		12.27	5.81	0.43	5.39	1.81	8.00	39.70
	Hachijo Is., Tokyo		13.58	6.63	1.58	5.93	1.64	3.00	48.00
	Ooe, Kyoto		12.01	4.86	2.63	4.00	0.98	4.00	30.80
	Ooe, Kyoto		14.53	6.82	2.95	5.15	1.12	4.00	42.43
	Aki, Hiroshima		9.51	5.35	1.30	3.63	0.52	3.00	23.29
	Toujyo, Hiroshima		8.52	3.65	1.21	3.93	0.56	4.00	14.30
	Yaku Is., Kagoshima		12.17	6.23	1.69	4.05	1.40	8.00	30.69
	Yaku Is., Kagoshima		15.46	6.88	2.31	4.94	1.86	7.00	40.47
	Yaku Is., Kagoshima		10.66	5.45	1.14	3.93	1.57	4.00	28.59
	Yaku Is., Kagoshima		12.18	5.23	1.76	4.60	1.53	8.00	31.37

N: number of individuals